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by V.A. Berezovsky, E.A. Goida, I.O. Mukalov, B.S. Sushko

AN EXPERIMENTAL STUDY OF OXYGEN DISTRIBUTION IN *Misgurnus fossilis* EGGS

The concentration of oxygen in animal cells is maintained by two main mechanisms: oxygen consumption and oxygen supply. As the rate of oxygen consumption increases, oxygen is supplied to the cell in greater quantities. According to the theory of diffusion, this is due to an increase in the concentration gradient in the cell, which leads to greater mass transfer of oxygen. It has been found that the conditions assuring oxygen supply may vary. Thus, the cells and tissues of living organisms are not homogeneous with respect to oxygen transport, but complex and compositionally and structurally heterogeneous. Oxygen supply and distribution may be largely dependent on the permeability of the plasma membrane to oxygen and on the rate of mass transfer of the latter within the cells.

We studied the distribution of oxygen in eggs of Misgurnus fossilis, the permeability of the membranes to the flow of oxygen and mass transfer of the latter in the eggs.

Method

Oxygen distribution in the eggs was determined by polarography using platinum microelectrodes with a tip diameter of 3-5 micrometres [8]. The permeability of the membranes to oxygen and the rate of mass transfer of the latter in the eggs were determined by comparing the experimentally registered oxygen tension profile (P_{O_2}) produced by an egg with the theoretical oxygen concentration profile calculated in accordance with the laws of oxygen diffusion [22].

The eggs used in the experiments were obtained by preliminary stimulation of the female fish with gonadotropin at the rate of 120-140 units per female. The eggs were fertilized artificially [3].

When the eggs enter fresh water the vitelline membrane separates and a perivitelline space forms between the yolk and cytoplasm [sic]. When the egg respires, oxygen enters it through the outer mucopolysaccharide membrane, then passes through the homogeneous perivitelline space, and, finally, through the inner envelope including the plasma membrane, thus entering the cytoplasm, where it is consumed.

The separated vitelline membrane together with the perivitelline space protects the egg cytoplasm against environmental convective disturbances, and in the course of the experiments conditions are created in which oxygen diffuses into the cytoplasm.

For the measurements we used both unfertilized and fertilized eggs. Their diameter averaged 1.8 mm, 1.2 mm of which was cytoplasm. The fertilized eggs had a larger perivitelline space than the unfertilized ones, but were virtually the same size.

In the experiments the eggs were placed on a coarse-meshed screen (1 x 1 mm) inside a deep non-flow-through chamber with a thin glass side-wall for optical control. The chamber was connected by an agar bridge to an external silver-silver chloride reference electrode. The working platinum microelectrode for stereotactic determination of P_{O_2} under microscopic control was positioned at certain points above the egg or inserted into it to the necessary depth. The experimental oxygen tension profile was constructed on the basis of the recorded diffusion current (I_d) proportional to P_{O_2} at 7 points. The first measuring point was well away from the egg, corresponding to the oxygen concentration in the surrounding water; the second near the surface of the egg; the third directly under the outer envelope as it was pierced; the fourth in the center of the perivitelline space; the fifth point above the inner envelope and the membrane separating the perivitelline space from the cytoplasm; the sixth, at the surface of the inner envelope as it was penetrated; the seventh in the centre of the egg. The measurements and the storage of the eggs were carried out at the temperature of the incubating medium (river water), i.e. 21-22°C.

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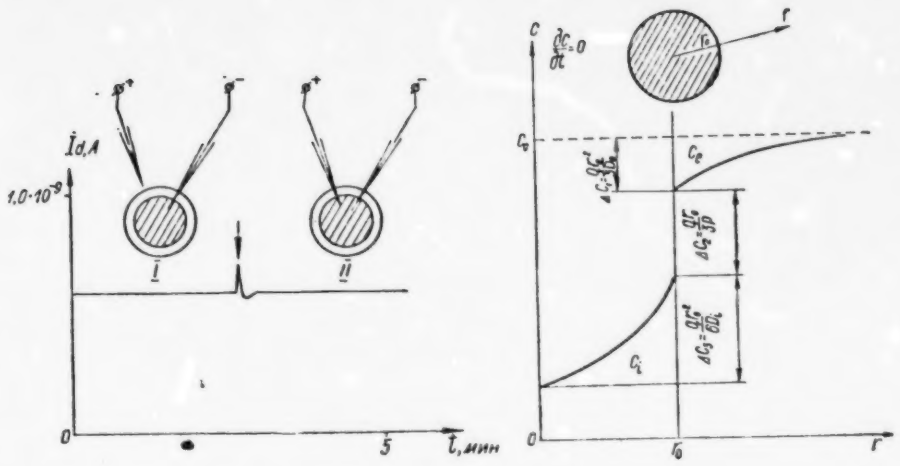

Fig. 1. Effect of the position of the reference microanode (+) on the oxygen diffusion current (I_d) in the egg.

I - part of the chronoamperogram with external microanode; II - part of chronoamperogram with internal microanode. The arrow indicates the moment at which the reference electrode was inserted into the egg.

Fig. 2. Theoretical oxygen concentration profile outside and inside a spherical cell.

Vertical axis - oxygen concentration, horizontal axis - distance from central cell. r_0 - radius of cell.

The microelectrodes for measuring the oxygen were stabilized with respect to the readings by means of electrochemical and biological ageing [1]. The microelectrodes were calibrated in two samples of river water of known oxygen content. The diffusion current was measured by the chronoamperometric technique. The polarographic cell voltage was 0.65 V. The diffusion current proportional to the oxygen tension was amplified (by a type OP-925, Radelkis amplifier) and registered on a KSP-4 recording potentiometer. Depending on the size of the active surface of the working part of the microelectrode the current sensitivity was 10^{-11}-10^{-10} A· $torr^{-1}$.

In order to minimize the traumatization of the cell, the reference electrode should be placed outside the egg. To test the legitimacy of this method we used a microanode that could be placed either outside or inside the egg. The reference microanode was a glass micropipette filled with 2.8 M KCl, which was connected with the electrical circuit via a chlorinated silver wire. The investigation revealed (Fig. 1) that when the oxygen diffusion current is measured inside the cell, the position of the reference electrode (inside or outside the egg) has no effect on the magnitude of I_d. This is evidently due to the high resistance of the platinum microcathode in the measuring circuit. At a current strength of 10^{-9} A, which is close to the maximum values of I_d, the resistance of the working electrode is 650 MOhms.

The theoretical oxygen concentration profile for the Misgurnus fossilis eggs was constructed on the basis of the diffusion theory of oxygen transport under steady state conditions of diffusion [22]. For a spherical cell with a radius of r_0 situated in an unmixed medium with an oxygen concentration of c_0, the equations for the distribution of the oxygen concentration in the cell (c_i) and in the solution above the cell (c_e) have the form: p. 381

$$c_i = c_0 + \frac{qr_0}{3P} + \frac{q}{6D_i}(r_0^2 - r^2) + \frac{qr_0^2}{3D_e} \qquad (1)$$

$$c_e = c_0 + \frac{qr_0^3}{3D_e}\frac{1}{r}. \qquad (2)$$

where r is the distance from the center of the cell; q is the oxygen consumption rate per unit volume of the cell ($q < 0$); D_i and D_e are the coefficients of oxygen diffusion inside the cell and in the surrounding medium; P - is the coefficient of cell membrane permeability.

The oxygen concentration profile with arbitrarily selected values of P, q, D_e and D_i corresponding to equations (1) and (2) is depicted in Fig. 2, from which it follows that the differences between the oxygen concentration at the remote point in the surrounding water and the concentration near the surface of the cell (Δc_1), on either side of the membrane (Δc_2), and the difference in oxygen concentration between the inner surface of the membrane and the surface of the cell (Δc_3) are equal respectively to:

$$\Delta c_1 = \frac{qr_0^2}{3D_e}, \tag{3}$$

$$\Delta c_2 = \frac{qr_0}{3P}, \tag{4}$$

$$\Delta c_3 = \frac{qr_0^2}{6D_i}. \tag{5}$$

Knowing the coefficient of oxygen diffusion in the surrounding water and the radius of the cell, and using system of equations (3-5), we can determine the rate of oxygen consumption, the permeability of the plasma membrane and the coefficient of oxygen diffusion inside the cell for experimentally measured differences in oxygen concentration Δc_1, Δc_2, Δc_3.

Results

In the first series of experiments the diffusion current was measured immediately after the fish had deposited the eggs in river water equilibrated with air. Owing to the higher concentration of oxygen in the river water and in the internal medium of the fish, the amount of oxygen inside the eggs increased for a while. One minute after an egg had been in the external medium (P_{O_2} = 150 torr), the oxygen tension in its center corresponding to the measured diffusion current did not exceed 10-15 torr. Subsequently, P_{O_2} in the egg grew; after 5 minutes P_{O_2} was already 40-45 torr, but it stabilized within 15-20 minutes after the vitelline membrane had separated and the perivitelline space had formed. I_d readings taken on fertilized eggs during 5 hours of development fail to reveal any substantial differences in the oxygen tension inside the egg compared with the unfertilized eggs.

The results of the investigations showed that the distribution of P_{O_2} in the eggs is symmetrical relative to the center. The only exception was the area of the cytoplasmic protuberance at the level of the perivitelline space, due to the asymmetry of that area.

When the microelectrode was brought closer to the surface of the egg the diffusion current diminished slightly, corresponding to a drop in oxygen tension of 5-6 torr. When the electrode penetrated the outer envelope of the egg, there was no accompanying change in I_d,

i.e. the difference in P_{O_2} in this area proved to be negligibly small (less than 1 torr). As the electrode penetrated deeper the drop in P_{O_2} p. 382 became greater, which showed that the P_{O_2} gradient increased with depth of penetration. Near the surface of the inner envelope and the plasma membrane the oxygen tension was on average 31 ± 2 torr lower than in the surrounding medium. In the region of the cytoplasmic protuberance this drop was 1-2 torr less. The greatest difference in P_{O_2} was recorded on the inner envelope separating the cytoplasm and the yolk of the egg from the perivitelline space. The average oxygen tension drop on it was 33 ± 5 torr. The difference in P_{O_2} inside the cytoplasm (from the inner surface of the membrane to the center of the egg) was equal to only 4 ± 2 torr. The oxygen tension in the center of the egg varied from 80 to 90 torr, which averages out at 85 ± 3 torr. Fig. 3 shows the experimental composite profile of P_{O_2} (oxygen concentration) of the egg equilibrated with air.

As the rate of oxygen consumption increased, the oxygen tension at the measuring points in and above the surface of the egg dropped. In order to increase the respiration rate after the formation of the perivitelline space, the egg was placed in a medium containing 10^{-4} M 2,4-dinitrophenol. Fig. 4 shows the dependence of the oxygen consumption rate on the incubation time of the eggs in the solution of 2,4-dinitrophenol and on the development time of the eggs. The data were obtained in direct experiments to measure the respiration rate of the eggs in a polarographic cell. At first respiration is stimulated, but after the eggs in the 2, 4-dinitrophenol solution have been

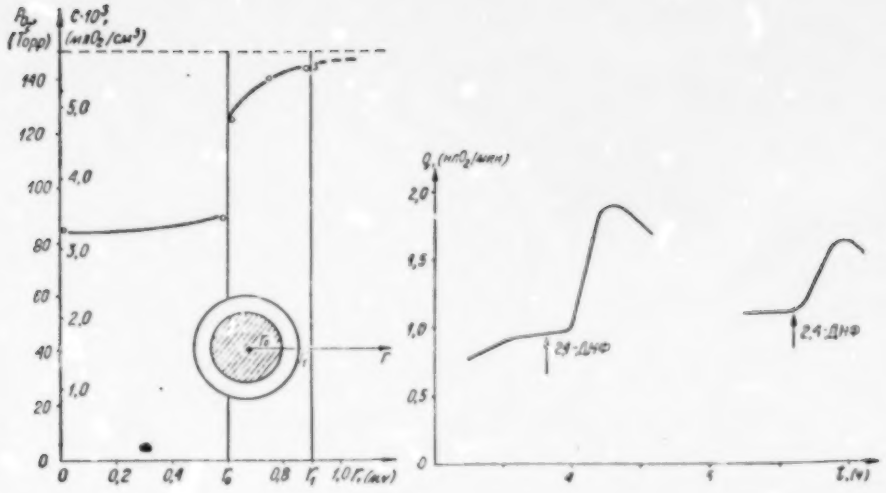

Fig. 3. Composite experimental profile of oxygen tension (concentration) of egg.

r_0 is the distance from the center of the egg to the inner envelope, r_1 is the radius of the egg.

Fig. 4. Graph of oxygen consumption rate versus time of development of egg with stimulation of respiration by 2,4-dinitrophenol.

The vertical axis shows the oxygen consumption rate per egg, the horizontal axis the development time. The arrow marks the moment of the onset of incubation of the eggs in a 10^{-4} M solution of 2,4-dinitrophenol.

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exposed for ½ hour it begins to decline. For this reason, we selected for investigation 2 groups of eggs in the first few hours of development which had been treated with 2,4-dinitrophenol for 15 and 30 minutes respectively. Table 1 shows the results of this investigation. p. 383

As the oxygen consumption rate increases, so also does the P_{O_2} gradient. 15 and 30 minutes after the beginning of exposure to 2, 4-dinitriphenol, the P_{O_2} values in the center of the eggs drop and

average 64 ± 5 and 17 ± 8 torr respectively. Because the oxygen tension in the surrounding medium remains constant, the total drop in P_{O_2} (the difference in the oxygen tensions between the external medium and the center of the egg) increases. The P_{O_2} profiles also alter after exposure (Fig. 5). A significant drop in P_{O_2} at the surface of the egg (near its outer envelope) was observed only for eggs treated with 2,4-dinitrophenol for 30 minutes. The drop in oxygen tension on the outer envelope when the oxygen consumption rate was increased remained small as before. This may indicate the outer envelope does not offer any significant resistance to the flow of oxygen. An increase in P_{O_2}, as intact eggs as well, is observed only on the inner envelope adjoining the cytoplasm.

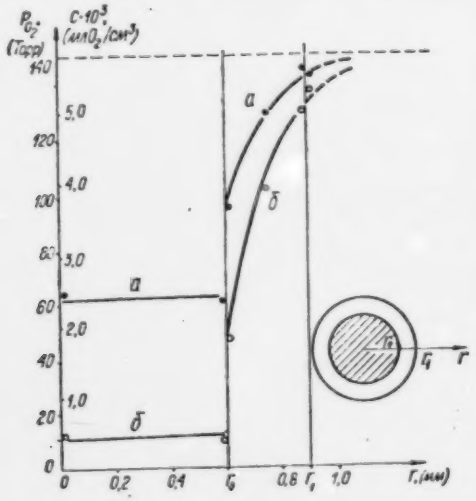

Fig. 5. Composite oxygen tension (concentration) profiles of eggs incubated for 15 minutes (a) and 30 minutes (b) in 2, 4-dinitrophenol.

For conventions see Fig. 2.

Table 1

Oxygen tension at fixed measuring points near intact Nisgurnus fossilis eggs after exposure for 15 and 30 minutes in a 10^{-4} M solution of 2,4-dinitrophenol

Item	P_{O_2} measuring points (torr)						
	1	2	3	4	5	6	7
Intact eggs	150±1	144±1	144±2	141±2	125±2	89±2	85±3
Eggs in 2,4-dinitrophenol for 15 minutes	150±1	143±1	145±3	129±5	95±12	61±9	64±5
Eggs in 2,4-dinitrophenol for 30 minutes	150±1	147±1	129±1	101±7	47±3	15±7	17±8

The average values of the oxygen tension differences corresponding to the oxygen concentration drops Δc_1, Δc_2, Δc_3 are shown in Table 2. The difference in P_{O_2} between the surrounding medium and the surface layer of the inner envelope ($\Delta P^1_{O_2}$) increased as the period of immersion of the eggs in 2,4-dinitrophenol lengthened. On the inner envelope the oxygen tension gradient remained almost unchanged when respiration was stimulated, and this was reflected in the constancy of the value of $\Delta P^2_{O_2}$, i.e. the differences of P_{O_2} on

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either side of the inner membrane. Inside the cytoplasm there was no drop in the value of P_{O_2} in these cases ($\Delta P^3_{O_2} = 0$); the oxygen tension is practically the same throughout the entire mass of cytoplasm.

When respiration is stimulated against the background of an increase in the total oxygen tension drop in the eggs, the proportion of $\Delta P^2_{O_2}$ responsible for the drop in P_{O_2} on the inner envelope declines. Whereas for intact eggs it was 54%, for eggs treated for 30 minutes with 2, 4-dinitrophenol it dropped to 24%.

Table 2

Average values of the oxygen tension difference ($\Delta P^1_{O_2}$, $\Delta P^2_{O_2}$, $\Delta P^3_{O_2}$), the oxygen consumption rate (q), the permeability of the inner envelope (P) and the coefficient of mass oxygen transfer in cytoplasm for Misgurnus fossilis eggs

Item	$\Delta P^1_{O_2}$ (torr)	$\Delta P^2_{O_2}$ (torr)	$\Delta P^3_{O_2}$ (torr)	$qx10^5$ (ml O_2/ $cm^3 \cdot c$)	$Px10^4$ (cm/c)	$D\ x10^5$ (cm/c^2)
Intact eggs	31±2	35±5	4±2	2.0±0.1	2.9±0.2	-
Eggs in 2,4-dinitrophenol for 15 min.	55±18	34±9	-	3.5±1.2	5.4±0.3	-
Eggs in 2,4-dinitrophenol for 30 min.	103±1	32±6	-	6.6±0.6	10.7±1.0	-

Remarks: $\Delta P^1_{O_2}$ is the drop in oxygen tension in the perivitelline space; $\Delta P^2_{O_2}$ is the drop in oxygen tension on the inner envelope; $\Delta P^3_{O_2}$ is the drop in tension inside the cytoplasm.

Assuming that the oxygen distribution coefficients in the surrounding medium and the egg were approximately equal, we calculated on the basis of the differences in oxygen concentration - Δc_1, Δc_2, Δc_3 - the rates of oxygen consumption per unit volume of cytoplasm (q), the coefficients of permeability to oxygen of the inner membrane (P) and the coefficients of oxygen diffusion in the cytoplasm (d_i) using formulas (3-5). Table 2 shows the values obtained.

When the eggs were exposed to 2,4-dinitrophenol for longer periods, the rate of oxygen consumption calculated on the basis of diffusion theory increased. The estimated value of q proved to be close to the values obtained in the direct experiments to measure the respiration rate of the eggs in the polarographic cell.

The coefficient of permeability of the inner envelope to oxygen was relatively low in the case of intact eggs, its average value being $(2.9 \pm 0.2) \times 10^{-4}$ cm x c^{-1}. When respiration was increased 3.3 times, the permeability of the inner envelope to oxygen increased 3.7 times. Calculation of the coefficient of oxygen diffusion in the cytoplasm of the intact eggs showed that D_i was far higher than the coefficient of oxygen diffusion in an aqueous solution at the same temperature. The average value for intact eggs was $(7.7 \pm 1.2) \times 10^{-5}$ cm^2 x c^{-1}. Owing to the absence of an oxygen concentration gradient, it proved impossible to calculate the coefficient of oxygen diffusion in the cytoplasm of eggs with an increased respiration level. p. 385

On the basis of the P_{O_2} profile measured in the experiments it was found that the distribution of oxygen in different parts of the egg

differs from its diffusional distribution in a spherical homogeneous body ("the ideal cell"). There is virtually no P_{O_2} gradient on the outer envelope of the egg even when the flow of oxygen is increased by stimulating respiration. At the same time, the considerable drop in P_{O_2} proved to be concentrated on the inner envelope and plasma membrane adjoining the cytoplasm. The P_{O_2} distribution profile obtained on real eggs does not correspond to the theoretical profile predicted by the diffusional theory of oxygen molecule transport in a homogeneous and stationary medium.

Discussion of Results

It is now known that diffusional transport of oxygen is not the only method possible in living systems. There are theories of facilitated oxygen diffusion based on passive transport of oxygen by various carriers [16, 18, 20], and indirect evidence of convective transport of oxygen in the cytoplasm of large cells [5, 6, 19]. Opposing views are expressed on the role of the envelopes and the plasma membrane in the transport of oxygen into the cell. Some investigators consider that the cell membrane is not a significant barrier to oxygen in an aqueous medium [13, 14]. Others prove experimentally and theoretically that the membrane offers great resistance to the diffusion of oxygen molecules [4, 10, 11].

Results of the investigations carried out by us show that in the layers of the aqueous medium adjoining the egg, and in the perivitelline space, oxygen is transported by diffusion. When this occurs, the outer envelope has no effect on the distribution of oxygen in these areas. On the outer envelope, however, there is a sharp drop in P_{O_2}. According to the diffusional theory, such a distribution must be due to the fact that there is no resistance to the flow of oxygen on the outer membrane, whereas on the inner envelope and the plasma membrane conditions are created for impeded passage of the oxygen molecules relative to an aqueous medium. If only diffusional transport of oxygen took place and there were no resistance on the inner envelope of the egg, instead of a marked drop in P_{O_2} there would simply be an increase in the oxygen tension gradient in the cytoplasm (as the result of uptake of oxygen).

There may also be a large drop in P_{O_2} on the inner envelope when the contents of the cell are vigorously agitated, even in the absence of substantial diffusional resistance to oxygen on the part of the envelopes and plasma membrane of the cell. It was shown experimentally that the cytoplasm of many large cells exhibits cyclosis, i.e. a state of continuous intense circulation. It follows from this that the existence of a high P_{O_2} gradient on the inner envelope of the egg may be due to both resistance on the part of the membrane to the flow of oxygen, and to active circulation of the cytoplasm.

A considerable difference in P_{O_2} on the inner envelope of the egg and low values of the P_{O_2} gradient in the cytoplasm are not only found in the species under study. For example, in an aqueous medium equilibrated with atmospheric air (P_{O_2} = 150 torr), the drop in oxygen tension on the membrane of sea urchin (Echinorachius mirabilis) eggs was 70-80 torr [10], whereas on the membrane of chicken egg cells it reached values of over 140 torr [9]. The drop in P_{O_2} on the membrane of mammalian muscle cells as a function of the P_{O_2} of the intercellular medium was over 50 torr [23].

Calculations based on the diffusional theory revealed that the permeability of the inner envelope to oxygen is fairly low; the permeability coefficient does not exceed 3×10^{-4} cm x c^{-1} for native eggs. The literature contains data showing that the plasma membrane of cells constitutes a substantial barrier to oxygen. Thus some investigators give values of 10^{-10}-10^{-11} cm_2 x c^{-1} for the coefficient of oxygen diffusion in the plasma membrane [4, 6].

In a study of gaseous diffusion across various thin organic films it was demonstrated that the permeability of the films is largely dependent on the structure of their material. The more ordered the molecules and the longer the chains of molecules, the less permeable they are to gases [7]. The far greater resistance to oxygen offered by the cell plasma membrane makes a certain amount of physiological sense. Because of the varying permeability of living tissue to oxygen, oxygen molecules can reach the cells closest to and furthest from the capillaries in more even quantities [17]. There is also a theory that

the reaction order for the uptake of oxygen by tissue is a function of the permeability of the cell membranes to oxygen [11]. The oxygen diffusion coefficient values known at present, which more properly represent the total coefficient for the entire thickness of tissue and ignore its heterogeneity, exhibit a greater degree of scatter, i.e. from 10^{-4} to 10^{-8} $cm^2 \times c^{-1}$ [21].

Presenting some interest are the results of investigations of D_{O_2} carried out on erythrocyte "ghosts" and artificial membranes [14]. Using the optical method, based on pyrene fluorescence extinction by oxygen, the authors arrive at the conclusion that the membranes do not constitute a barrier to oxygen. In our view, one of the deficiencies of these experiments is that the molecules of oxygen need only enter the phospholipid membrane in order to cause extinction of fluorescence. Nevertheless, it may be difficult for the oxygen to pass into solution from the phospholipid layer, where it dissolves more readily [2].

There are other possible explanations for the significant P_{O_2} difference recorded on the inner envelope of the egg. The first possibility is that the oxygen absorption coefficient varies from one point of measurement to another. However, it is difficult to attribute the large drop in P_{O_2} to differences in the distribution of oxygen inside and outside the cell. It is known that oxygen is several times more soluble in the lipid elements of the cell than in water [12], but the quantity of these lipid elements is insignificant, since water accounts for approximately 80% of the weight of the cell. A sharp drop in the diffusion current on the inner envelope of the egg may also

occur when the ohmic resistance in the polarographic circuit contributed by the membrane itself increases. To test this hypothesis special experiments were run using a reference microelectrode placed in the egg. Insertion of the microanode beyond the inner envelope of the egg had no effect on the value of the diffusion current. p. 387

Calculations based on the assumption that oxygen is transported by diffusion alone indicate that the oxygen permeability coefficient of the inner envelope varies. It increases when the respiration of the egg is stimulated. When respiration was increased by a factor of 3, the drop in P_{O_2} on the inner envelope remain unchanged, which is equivalent to an increase in the permeability of the envelope to oxygen. Tests with chicken egg cells also revealed a change in the value of P for the envelope. In addition, permeability to the flow of oxygen entering the cell diminished when the oxygen uptake rate was partially inhibited by cyanide [9]. At present it is not easy to identify the causes of the change in cell envelope permeability. It seems likely that permeability to oxygen may be connected with changes in the diffusional resistance of the envelopes and in the rate of cytoplasmic streaming against a background of a varying rate of oxygen uptake.

The small drop in P_{O_2} discovered in the cytoplasm of Misgurnus fossilis eggs suggests that after the oxygen has diffused through the inner envelope it is very rapidly transported into the egg. In intact eggs the calculated diffusion coefficient is 7.7×10^{-5} $cm^2 \times c^{-1}$,

while in some cases, even after exposure to 2,4-dinitrophenol it approaches far higher values. In known experiments to study the respiration of tissue slices of varying thicknesses and on the basis of measurements of the values of critical P_{O_2}, it was shown that D_{O_2} is 3-7 times greater in liver, miocardium and kidney tissue than in water. Hence the authors put forward the hypothesis of the existence of "active intracellular transport of oxygen", i.e. convective transfer of oxygen by the streaming cytoplasm [19], while in later works they presented a number of proofs of the possibility of passive mediated transport of oxygen in tissue and cells [15, 20].

The results of our experiments also confirm that the distribution of oxygen in the cytoplasm of Misgurnus fossilis eggs takes place much more rapidly than in an aqueous unmixed medium. The high diffusion coefficient of oxygen in the cells together with the phenomena of cyclosis should be regarded as due to a combination of diffusional and hydrodynamic transport of oxygen. In that case, the term "diffusion coefficient of oxygen" should be replaced by the term "transfer coefficient of oxygen". The magnitude corresponding to this term appears to describe very adequately the transport of oxygen in cells and living tissue where, in addition to diffusional and convective transfer, facilitated diffusion of oxygen is also possible.

Conclusions

1. Direct polarographic measurements revealed that between the outer medium equilibrated with air and the central area of the

spherical Misgurnus fossilis egg there is a drop in P_{O_2} of approximately 65 torr which, when respiration is stimulated by 2, 4-dinitrophenol, increases to more than 130 torr.

2. No drop in P_{O_2} is registered on the outer envelope.

3. The greater part of the oxygen tension drop (54%) occurs on the inner envelope of the egg, which is evidently a diffusional barrier to oxygen molecules. When respiration is stimulated with 2,4-dinitrophenol, the drop in P_{O_2} on the inner envelope does not change. p. 388

4. The coefficient permeability of the inner envelope of intact eggs to oxygen, calculated on the basis of the theory of diffusion, proved to be equal to $(2.9 \pm 0.2) \times 10^{-4}$ cm x c^{-1} and increased as the rate of oxygen uptake by the eggs rose during stimulation of respiration by 2,4-dinitrophenol.

5. In different parts of the egg cytoplasm, the oxygen tension was practically the same.

6. The higher values of D_{O_2} in the cytoplasm of the egg compared with D_{O_2} in water may be the consequence of a combination of diffusional and hydrodynamic transfer of oxygen.

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